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Resistant starch in the diet of rodents promotes an increase in fermentation and a reduction in body fat in an animal model of dietary obesity

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RESISTANT STARCH IN THE DIET OF RODENTS PROMOTES AN INCREASE IN
FERMENTATION AND A REDUCTION IN BODY FAT IN AN ANIMAL MODEL OF DIETARY
OBESITY

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
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By
Jason Charrier
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ABSTRACT

In previous studies, it has been observed that the addition of resistant starch (RS), a non-digestible fermentable fiber, in the diet of rodents promotes an increase in fermentation and decreases in body weight and body fat when incorporated into low to moderate fat diets. This study investigated whether the same beneficial effects observed with RS addition in these lower fat diets could be obtained in a high fat diet, and if the type of fatty acid consumed in the diet makes a difference in markers of fermentation and body fat. Sprague Dawley rats (n=96) were fed as weight of diets, RS (27%), and Hi-fat (20%) or low fat (7%), for 12 weeks. Fish oil (4%) was given to half of the groups in order to assess the effects of fatty acid composition. Markers of fermentation (pH and SCFA) were significantly decreased and increased, respectively, in RS fed groups ($p < 0.05$), and the high fat diet partially interfered with these improvements by reducing the amount of fermentation when compared to low fat groups. Gut hormones glucagon like peptide-1 (GLP-1) and peptide YY (PYY) were also significantly increased ($p < 0.05$), but high fat diet demonstrated partial interference with PYY levels by reducing the level of this hormone when compared to low fat fed groups. Body fat was decreased in RS fed animals ($p < 0.05$), but the reduction was reduced from 24% in low-fat fed animals to 9% in high-fat fed animals. In conclusion, RS promotes benefits in fermentation and body fat reduction, but the fat content of the diet moderated the level of improvement observed.

CHAPTER 1: INTRODUCTION

Significance of Research

The incidence of the overweight and obese individuals in the American population has grown to epidemic proportions. It is estimated that greater than 60% of the adult population is overweight or obese, as measured by a BMI ($\text{kg body weight}/\text{height}^2$) of 25. 30 and 30+, respectively. The co-morbid conditions associated with obesity include diabetes mellitus, hypertension, dyslipidemia, and cardiovascular disease. Estimates of the economic burden associated with obesity in medical cost are greater than \$147 billion a year in the United States alone (dept. of HHS 2009).

As the burden of obesity becomes even more apparent, the effort to develop food products to reduce overweight and obesity as a relatively low-cost option has gained widespread attention in the scientific community. The result has been increased efforts to develop functional foods, which can easily be added to the common American diet, which may cause reductions in the incidence of obesity. Functional food is any healthy food claimed to have a health-promoting or disease-preventing property beyond the basic function of supplying nutrients (Shamrock LLC). Common examples of functional foods include adding DHA to butter to lower cholesterol, added fiber to bread products, and the addition of prebiotics in yogurt to promote a healthier colon. The “Western diet” of Americans is typically high in saturated-fatty acids and calories, and low in fruit and vegetable consumption. The average fat intake in the American diet is in the range of 35-40% of kcalories (Kennedy et al 1999). While efforts to reduce the caloric energy density of the diet and to increase physical activity have been largely proven to be ineffective at reducing the rates of obesity, efforts are currently being shifted to a multi-factorial approach to

reduce obesity, which incorporates many small changes which are easier to follow. The addition of functional food components to the diets of Americans may prove to be more effective than large-scale efforts to promote weight loss and co-morbid conditions associated with obesity. Resistant starch (RS) is a functional food component, which may offer benefits in reducing body fat and increasing beneficial markers of gastrointestinal health.

Objectives

The objectives of this study were to investigate the effectiveness, in a rodent model, of a fish oil supplementation and RS addition in high-fat and low-fat diets to assess gut fermentation, body weight analysis, and gut hormone involvement. The study utilized rodents, as rats, for both the control and treatment groups. Rodent studies are common mechanisms for conducting preclinical trials before human studies that are then necessary to validate the results. The use of rats as the class of rodents allowed for greater sample collection when compared to smaller rodents, such as mice.

Hypothesis:

The addition of RS in low and high-fat diets of rodents will result in significant improvements in markers of fermentation and body composition in low-fat and high fat-diets. Gut fermentation will be measured by ileal, cecal, and colonic pH, SCFA production, and specific concentrations of SCFA's acetate, propionate, and butyrate. Based on the results of previous research, the addition of RS to the diet results in decreased body fat, and increased fermentation.

Fish oil supplementation will work in a synergistic fashion with RS in order to promote fermentation and a decrease in body weight and body fat, which may be lost due

to a high-fat diet. It is our hypothesis that the addition of fish oil and RS in the diet will demonstrate an increase in fermentation and a reduction in body fat in high fat diet with RS when compared to high fat control diets without RS. In previous studies, fish oil and a low dietary dose of RS inclusion demonstrated a synergistic effect on increasing SCFA production and decreasing colonic (cecal) pH compared to the control diet (Vidrine et al 2009). These findings have led to the hypothesis that an addition of fish oil will produce significant changes in body weight and body fat. These results also suggest an improvement in the fermentation effects of RS with fish oil.

It is our hypothesis that the addition of RS to the diet will result in increased levels of hormones total GLP-1, GLP-1 active, and PYY. In previous research, RS intake has been associated with increased gut hormone response.

Limitations

In order to be able to draw conclusions of comparisons between RS groups/fish oil groups and control groups, energy balance must be controlled across groups. This can only be done across low fat and high fat diets separately. Thus, there is the need for appropriate controls within high and low fat diets in a factorial design in this study. It is not possible to control for both non-fermentable and fermentable fiber sources (total fiber) among the diets, therefore, for this study we controlled for dietary energy and non-fermentable fiber and tested the effects of different levels of fermentable fiber on markers of fermentation, body weight/fat, and gut hormone levels.

This study is based on the assumption that the animal model results, in most aspects of importance to the study, are similar to the results in humans. Animal studies are common methods of preclinical and mechanistic trials before the administration of human studies.

This may present problems of generalizability of prebiotic effects from animal to applicability in human subjects.

The percentages of RS and fish oil used in the study are based on past studies in which beneficial effects were observed (Vidrine et al 2009). However, the levels of dietary RS was increased from 20 to 27% as we most consistently observe results of decreased body fat when dietary RS is greater than 25% of the weight of the diet (Keenan et al. 2006, Shen et al. 2009, Zhou et al. 2009). The use of 27% of the diet as RS is not feasible in a human diet and, therefore, questions if an amount acceptable in a human diet will be as effective. Likewise, fish oil is administered at 4% of the diet by weight, which also may not be a useable amount in a human diet. Nevertheless, preclinical trials involving bioactive food components are typically conducted to study the mechanism of action before human trials are necessary. By allowing for the mechanism of action to be understood before the human trails are conducted may allow for the use of smaller amounts of the food components which act through a similar mechanism observed in the preclinical animals trials.

CHAPTER 2: REVIEW OF THE LITERATURE

Fiber

The term “fiber” can often bring up many associated connotations from a broad spectrum of subjects. Narrowing down the topic of fiber into the term “dietary fiber”, results in less ambiguity as it relates to its effects on nutrition and chronic disease development. The term dietary fiber is defined as nondigestible carbohydrates and lignin portions of plants (Dietary Reference Intakes 2002). Together with functional fibers, which are isolated nondigestible carbohydrates, dietary fiber makes up the total fiber in the diet. The attention dietary fiber has received in the scientific community has grown exponentially in the past decades as more information has been elucidated concerning this dietary component. The research in dietary fiber reveals an inverse relationship between fiber consumption and prevalence for various chronic diseases such as coronary heart disease, stroke, hypertension, diabetes, obesity, and certain gastrointestinal disorders (Anderson et al 2009). Furthermore, the intake of fiber has also been found to have an inverse relationship with obesity. When moderately and severely obese individuals were compared to normal weight groups it was revealed that the normal weight groups consumed a significantly higher amount of dietary fiber than their obese counterparts (Alfieri et al 1995). The anomaly of fiber consumption within the American diet seems to be that as more benefits of fiber consumption become available, the average intake of dietary fiber has decreased. The current requirements for dietary fiber are 14 grams per 1000 kcalories daily, while the average consumption in the American diet appears to be approximately 2-3 grams per 1000 kcalories (National Academy of Sciences 1995). The most common food sources of dietary fiber include legumes, wheat, barley, fruits and vegetables, nuts, and seeds. The common “Western diet” of the American population is defined

as high-saturated fatty acid content, high in energy, high in starch and sugar, and low in dietary fiber, and fruits and vegetables (Mc-Graw Hill 2002).

Resistant Starch

Along with the high-saturated fatty acid content, a large portion of the common American diet consists of highly digestible carbohydrates in the form of starch. Starch is composed of two components, mainly the linearly structured units of glucose in amylose, and the highly branched units observed in amylopectin. The high branching structure of the amylopectin, in comparison to amylose, enables the amylopectin to be more readily digested (Nugent et al 2005). Resistant starch (RS) is a class of nondigestible carbohydrates, which is resistant to digestion in the small intestine, yet is fermented by the microflora of the colon (Zheng et al 2010). RS occurs mainly in one of four major types. The RS from type 1 is able to resist digestion by being protected and physically inaccessible to digestive enzymes. Common food sources of type 1 include whole grains, rice, seeds, and legumes. The second type of RS is found in a dense form of starch granule made up of amylose that is able to resist digestion by being tightly folded and packed which does not allow access to the glucose units. Food sources of type 2 RS include raw potatoes, bananas, and starches containing a high amylose content. Type 3 RS is a retrograded starch that is non-granular and becomes resistant to digestion after being cooked and cooled. Examples of RS type 3 would be cooked and cooled potatoes in potato salad, bread, or cereals. The last type of RS, type 4, is chemically modified starch that resists digestion through the alterations of its bond structure. Examples of type 4 RS only includes foods, which have been chemically modified. Hi-maize cornstarch is a type 2 RS, which has a high content of amylose, packed tightly in starch granules, which allow the starch to resist digestion by

amylase in the small intestine, and can be fermented by bacteria in the large intestine. In this study Hi-maize cornstarch (Hi-maize 260, National Starch LLC.) was used as the RS source. Information concerning RS types is from Topping, Fukushima, and Bird (2003).

It is believed that the fermentation of RS is associated with the beneficial effects observed in the addition of this fermentable fiber to the diet. In research, RS inclusion into diets and comparison to energy-controlled diets resulted in significantly lower total abdominal fat, decreased cecal pH, and increased concentrations of gut hormones GLP-1 and PYY (Keenan et al 2006, Zhou et al 2008, Shen et al 2009). RS is fermented in the colon by the microbial bacteria of the GI tract. This fermentation results in the production of beneficial short chain fatty acids (SCFA), mainly acetate, propionate, and butyrate (Nugent et al 2005). The production of SCFA that result due to the fermentation of RS is associated with an increase in concentration of GLP-1 and PYY in serum, as well as increased gene expression of these gut hormones (Keenan et al 2006, Zhou et al 2006, Zhou et al 2008).

The SCFA butyrate has been implemented in a wide range of benefits. Butyrate exerts anti-carcinogenic effects through decreasing growth of colonic tumor cells in vivo (McIntyre et al 1993). This result could be attributed to butyrate's ability to increase rates of apoptosis in malignant cell growth (Kolar et al 2007). Butyrate feeding has also been associated with increased energy expenditure as an anti-obesity target (Gao et al 2009). The benefits associated with RS consumption appear to rely on the ability of the fiber to ferment. In mice fed RS at 30% of the diet, which did not display markers of fermentation (decreased pH, SCFA production), no body fat loss was observed, but mice, which did display signs of increased fermentation, demonstrated a subsequent loss in body fat (Zhou et al 2009). It is important to note that the mice, which did not ferment the RS in the study,

were genetically obese mouse models. It appears that the incidence of obesity plays a significant part in the effectiveness of the fermentation of RS and on the resulting body fat losses. Mice which were made obese prior to the start of the study that were fed RS, demonstrated no loss in body fat or significant changes in markers of fermentation (Goldsmith et al 2010). These observed effects in obesity may be explained through the involvement of signaling pathways involving the gut hormones GLP-1 and PYY.

Glucagon-Like Peptide-1 and Peptide YY

Glucagon like peptide-1 (GLP-1) and Peptide YY (PYY) are gut secreted hormones that are released postprandially, and exhibit anti-obesity actions through their effects on energy metabolism and appetite regulation (Zhou et al 2009, Badman and Flier 2005). GLP-1 and PYY are secreted from the intestinal L-cells and the resulting plasma concentrations of these hormones increases following a meal (Neary et al 2005). The combined effects of peripheral administration of both GLP-1 and PYY result in an additive effect on food intake inhibition in the ob/ob model of obesity in mice and in human trials (Neary et al 2005). In human trials, PYY infusion demonstrated the same level of food intake suppression in obese versus lean subjects (Batterham et al 2003). These results suggest that PYY may be a more effective treatment for obesity than leptin administration because of the resistance to leptin typically demonstrated in obese subjects (Neary et al 2005). The gut fermentation that takes place with the ingestion of RS is associated with an increase in GLP-1 and PYY. The increases in GLP-1 and PYY after RS ingestion have been shown to be sustained in a daylong manner (Zhou et al 2008). This suggests that the effects of RS fermentation on GLP-1 and PYY are not an effect of meal ingestion or glycemic response of the diet. That may be the reason that increased serum GLP-1 and PYY associated with feeding of high

dietary doses of RS has not resulted in decreased food and energy intake (Keenan et al. 2006, Shen et al. 2009, Zhou et al. 2009). In support of this conclusion, repeated infusions of PYY into rats led to increased food intakes after several infusions despite reduced body fat in the rats (Reidelberger et al 2008).

Resistant Starch and Gastrointestinal Health

RS is defined as that which makes it to the large intestine where it can be fermented into short chain fatty acids (SCFA), which decreases the pH of the gastrointestinal (GI) milieu and promotes GI health (Nugent et al 2005). RS is able to resist digestion by mammalian enzymes, most notable amylase, but is fermented by the bacteria or microbiota of the digestive tract. The benefits of RS in gastrointestinal (GI) health have garnered an increasing degree of attention from the researchers and healthcare professionals recently (Grabitske et al 2009). The beneficial effects of RS on GI health can largely be attributed to the actions of SCFA's. Benefits observed from SCFA's and GI health include increased mucous barrier function, altered inflammatory and immune effects, increased immunities, and benefits in reducing certain types of cancers (Mortensen et al 1996). The three main SCFA's receiving the attention in terms of beneficial effects are acetate, propionate, and butyrate (Mortenson et al 1996). The functional effects of fermentable fibers, such as RS, are largely related to the SCFA production and absorption by the colonocytes. The primary effects of SCFA's in the colon are dependent on their metabolism and uptake by the colonocytes (Bergman et al 1990). The concentrations and amounts of specific SCFA depend largely on the microflora of the GI tract, which ferment the fibers. Each of the SCFA's exhibits different transport and modes of action (HiJova et al 2007).

Acetate is the chief SCFA present in the blood, and is commonly observed in the highest amounts in the GI tract (HiJova et al 2007). Acetate's higher concentrations in the blood indicate that it is less metabolized in the colon. It acts as the primary substrate for cholesterol synthesis. Acetate escapes through the liver and enters primarily into the systemic circulation to be metabolized by peripheral tissues of the body.

Propionate is rapidly taken up by the liver upon its production in animal studies (HiJova et al 2007). The knowledge of propionate absorption and actions is primarily based on studies in ruminants, and in the past less was known on the specific effects in humans (Cummings et al 1981). A recent review has summarized the effects of propionate in humans. The propionate which is produced by microbiota of the GI tract is rapidly absorbed and drains into the portal blood and bypasses the colonocytes (Al-Lahham et al 2010). Propionate is converted into propionic acid in the liver. In the liver, a large amount of the propionic acid is converted into glucose making propionate the primary source of glucose production among the short chain fatty acids (Lang et al 1963). Propionate has been associated with a reduction in GI inflammation through its inhibitory actions on cyclooxygenase enzymes, which are involved in pro-inflammatory markers (Bos et al 2004). This reduction in inflammation has implemented propionate as a possible therapeutic agent for improvements in insulin sensitivity (Harvesen et al 2009). The ratio of propionate to acetate appears to play a part in determining the beneficial effects of this SCFA.

Butyrate has received the largest attention among the SCFA because of its diverse actions. Butyrate is the major source of energy for the colonocytes (HiJova et al 2007), which can explain why an increase in butyrate production resulting from the fermentation

of fibers often results in a larger and healthier GI tract (Keenan et al. 2006). Butyrate is involved in regulation of apoptosis and cellular proliferation, which implicates this SCFA in cancer prevention (Zoran et al 1997). Gene expression can also be affected by butyrate through its effects on phosphorylation and acetylation of histone proteins (Archer and Hodin et al 1999). Butyrate is also implicated in the down-regulation of the genes in harmful bacteria virulence, and can increase cell proliferation in the host cells against these bacteria (Guilloteau et al 2009). Proliferative studies have demonstrated that necrotic areas in the colon experience a faster renewal with the addition of butyrate in cell cultures. The multiple roles of butyrate on colonocytes and their metabolism have increased the speculation of its far-reaching effects on GI health. Reductions in the production of butyrate, which are observed in the elderly population, could result in increased degenerative diseases of the colon and small intestine (Hippe et al 2011). Fermentable fibers, such as RS, which significantly increase the production of butyrate, could prove to be a viable treatment option in order to promote a healthier GI environment.

The benefits of RS in GI health caused by the increased SCFA production and healthier gut environment extend from improvements in diarrhea to evidence of colon cancer reductions. Aberrant crypt foci (ACF) demonstrate early morphological changes when induced with Azoxymethane (AOM) in colonic cells. The cells exhibit pre-neoplastic lesions and are characterized by increased size of the crypts, epithelial lining, and peripheral tissue (Velmurugan et al 2008). AOM-induced colonic ACF is an effective way to study the morphology of cancer cell lines due to the similarities to tumor cell growth. When rodent's diets were supplemented with RS after having AOM induction of ACF, RS fed animals had suppressed AOM-ACF at the promotion stage (Liu et al 2007). These actions

can, most likely, be explained by the actions of butyrate on the histone proteins of the tumor progression cells. The addition of RS may prove to be an effective supplemental treatment for cancer through its increase in SCFA production.

The GI tract is a dynamic and versatile site in which a large number of actions essential to health maintenance take place. A large number of these actions are only beginning to be understood and present a new mode of thinking in the effects of the GI tract interactions with the external environment. The immune system is present within the GI tract in the form of the mucosa-associated lymphoid tissue (MALT), which consists in part of mucus synthesized by the mucins of the epithelial cells (Turner et al 2009). Specialized epithelial cells in the GI tract secrete the mucins, which produce mucus for the formation of a selective permeability that allows the passage of small ions but excludes larger harmful bacteria (Johansson et al 2008). The largest surface area of MALT exists in the GI tract. These mucosal sites create a barrier between harmful antigens and bacteria and the underlying tissue, and this barrier function is critical in the immune system regulation. Altered barrier function often results in disease and inflammatory responses. Damage caused to the epithelial cell membrane in cells which secrete mucins is believed to be the cause of decreased immune function resulting from altered barrier function observed in some cytotoxic drugs in chemotherapy treatment (Turner et al 2008). The tight junctions that exist in the mucosal barrier allow for a controlled system of selective permeability. Tight junction barrier dysfunction in which the homeostatic relationship is affected is the cause of the proinflammatory response observed in a wide variety of inflammatory bowel diseases (Hollander et al 1988). While the barrier function may be involved in the onset of chronic disease, barrier dysfunction alone is insufficient to cause disease (Su et al 2009). The loss of barrier function can exacerbate conditions, but immunoregulatory responses

are often responsible for mediating the loss in barrier function. It is usually when individuals with a preexisting immunoregulatory disease experience barrier dysfunction that conditions worsen. Decreases in anti-inflammatory markers, such as Interleukin-10, have been observed in mice, which exhibit barrier dysfunction (Baird et al 2008).

The importance of the mucosal barrier function in chronic disease is becoming better understood and presents an additional benefit of fermentable fibers, such as RS, in promoting a healthier gastrointestinal tract. The intestinal mucosal thickness is positively correlated with SCFA concentrations, and experienced the greatest improvements in rats fed RS (Hedemann et al 2006). The presence of RS in the diet is effective at providing nourishment to the colonocytes in the form of the SCFA butyrate. This creates a larger surface area for the mucosal barrier function to exist, and results in improvements in the overall function of the immune system.

The ability of RS to be fermented into SCFA's is effective at creating a healthier GI environment through decreasing the pH, nourishing the colonocytes, improving mucosal barrier function, and offering benefits in the function of the immune system and inflammatory response. The future in RS research will likely include each of these characteristics and seek to provide further understanding of fermentable fibers and their effects on gastrointestinal health. Studies, which are dedicated to understanding the effect of RS in the diet on specific inflammatory markers, are needed to enhance the understanding of GI health at this point. The use of human trials should also be conducted to assess if these effects are affected by biological variance and genetic variations between individuals.

High-Fat Diet Effects

The increased fermentation and improvements in body composition and GI health observed with the addition of RS in the diet is typically observed in low to moderate fat

diets. When substantial amounts of fat make it to the large intestine they can interfere with the bacterial fermentation. The main reason for this effect is believed to be because of the antimicrobial effects of the unsaturated and saturated fatty acids (Ferguson et al 1990, Jenkins et al 1993). The fatty acids associate with the bacterial cell wall and disrupt the cell membrane of the bacteria (MacZulak et al 1981). The results from an *in vitro* assay reveal that high fat reduces beneficial bacteria (unpublished data). The results reveal that after 24 hours of incubation, the anaerobic bacteria population (inocula from rat ceca) expressed as the log colony forming units per gram show that RS is at 10, 2% fish oil with RS at 9.6, 2% corn oil with RS at 9.2, and 2% lard with RS at 9.0. These results demonstrate a significant reduction from fat, but fish oil demonstrates the least reduction. In *in vivo* studies a similar effect is observed. A 41% high-fat diet (percent of energy) with RS in mice revealed significant reductions in specific bacterial populations involved in the production of SCFA's (unpublished data). In contrast, low and moderate fat content diets with RS consumption demonstrated increased beneficial bacterial populations of bifidobacterium and lactobacillus, and increased markers of fermentation (unpublished data). The average fat intake in the American diet is in the range of 35-40% (Kennedy et al 1999), and this could reduce the effectiveness of certain functional foods, such as RS.

Fish Oil

The source of fat content of the diet may be of critical importance to regaining the beneficial gut fermentation in a RS diet. Fish oil has been shown to offer many benefits in cardiovascular health, and also exhibit anti-obesity effects (Li et al 2009). Fish oil inhibits enzymes, which are involved in the production of adipocytes and lipid synthesis. The DHA in the fish oil has been shown to increase thermogenesis, and to inhibit free fatty acids in

the blood- stream from being involved in lipogenesis (Arai et al 2009). Fish oil has also promoted increased insulin sensitivity in both animal and human studies (Kuda et al 2009). In animal models when fish oil was compared to safflower oil in mice, fish oil fed groups had inhibited weight gain, and lipid synthesis. Fish oil and RS fed rats also demonstrated increases in SCFA production, lower colonic pH, and higher n-3 fatty acid levels when compared to a control diet of sunflower oil and RS (Conlon et al 2009). In several studies comparing the effectiveness of different dietary lipid sources on gut fermentation, fish oil has been shown to be superior. Fish oil has also been shown to enhance the effect of the SCFA butyrate in apoptosis of colon cells by increasing mitochondrial lipid oxidation (Kolar et al 2007). In previous research, high fat diets containing corn oil at 35% of the diet as the fat source demonstrated increased weight gain and body fat, increased insulin resistance and decreased insulin response and an increase in adipocyte biogenesis which were reversed in diets which replaced 15% of the corn oil with fish oil (Patten et al 2006). In previous research, improvements in fermentation markers have been observed with the addition of fish oil and RS to the diet (Vidrine et al 2009).

CHAPTER 3: MATERIALS AND METHODS

Animals, Housing, and Diets

Ninety-eight male Sprague Dawley rats arrived at the LSU Veterinary School at age 4 weeks and were housed separately in shoebox cages in a climate-controlled environment (22 +/- 2°Celsius, 65-67% humidity) with a 12-hour light and dark cycle daily. Prior to the start of the study, rats were quarantined for a week with chow diets, then switched to hanging stainless steel wire bottom cages and acclimated to powder diets by consuming the low-fat control diet for a week. This was done to allow the animals to be accustomed to powder diet consumption and the hanging stainless steel wire bottom cage environment for the duration of the study.

Following the 2-week quarantine and acclimation period, at 6 weeks of age, rats were balanced according to weight and then assigned to one of the eight experimental diets. Treatment one consisted of the first 12 cages and this continued through 8 treatments/96 cages and extra rats shipped with the order. The mean of all rats +/- SD was determined. The extras furthest from the mean were removed. Then the treatments were balanced by moving rats among treatments. The balancing was completed when all of the means of the treatment groups were similar and the weights were normally distributed within treatment groups. General composition of the diet is shown in table 1, and the ingredients of the diet in table 2, while the complete diet table and ingredient list can be found in the Appendix: Diet Table and Mixing.

The experimental diets were energy-balanced within low fat and within high-fat categories so that all low-fat diets provided 3.5 kcal/gram and all high-fat diets provided 4.2 kcal/gram. It is not possible to balance for both fermentable and non-fermentable fiber within the diets, therefore, non-fermentable fiber was held constant at 15% of the diet, and we tested for fermentable fiber effect in the diet, as stated earlier. The fish oil content of both low and high fat

diets was added at 4% of the weight of the diet. Corn oil was used in the low-fat diets at 2% of the weight of the diet in order to supply adequate linoleic acid.

	LC	HC	LR	HR	LF	HF	LRF	HRF
Protein	14%	14%	14%	14%	14%	14%	14%	14%
Carbs	66%	52%	75%	61%	66%	52%	75%	61%
Fat	6%	20%	6%	20%	6%	20%	6%	20%
RS	-	-	27%	27%	-	-	27%	27%
FO	-	-	-	-	4%	4%	4%	4%

Table 1: General diet composition expressed as percent weight (g/100g) of diets
H=hi-fat, L=low-fat, R=Resistant Starch, F=fish oil, C=control diet

Ingredients	LC	LR	LRF	LF	HC	HR	HRF	HF
Amioca	531.9	171.9	171.9	531.9	391.9	31.9	31.9	
							391.9	
Hi-maize	0	480	480	0	0	480	480	0
Sucrose	100	100	72.5	72.5	100	100	72.5	72.5
Casein	140	140	127.5	127.5	140	140	127.5	
Corn oil	60	60	20	20	100	100	127.5	
Lard	0	0	0	0	100	100	80	80
Cellulose	120	0	0	120	120	0	80	80
Fish oil	0	0	83.3	83.3	0	0	0	120
							83.3	83.3
Mineral mix	35	35	32.5	32.5	35	35	32.5	32.5
Vitamin mix	10	10	9.2	9.2	10	10	9.2	9.2
Choline	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
L-cystine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
BHQ	0.012	0.012	0.012	0.012	0.04	0.04	0.04	0.04

Table 2: Diet Ingredients listed as g/kg * Fish oil composition can be found in the appendix (fish oil contains sugar, protein, vitamins, and minerals)

In the high-fat diets, the additional fat above the 4% fish oil was added at a 50:50 ratio of corn oil and lard to a level of 20% of the weight of the diet.

We utilized high and low-fat control diets as well as positive control diets for fish-oil content to allow for a complete 2X2X2 factorial design which provides greater statistical power than in a one-way ANOVA. Animals were allowed free access to food and water throughout the duration of the study. The experimental protocol was approved by the Louisiana State University Institutional Animal Care and Use Committee (IACUC #10-023). The approval letter can be found in the Appendix.

Methods and Measurements

The sacrifice of the animals took place over a four-day period due to the large number of rats used and the data collection techniques of the laboratory personnel. Animals were killed according to groups of 8 rats with one rat per group. Animals were supplied with food and water during the waiting period in between animals killed. Rats were anesthetized under isoflurane anesthesia prior to cardiac puncture. The heart was then removed to ensure death prior to dissection. Blood was collected into two tubes for each rat, one with aprotinin and DPP4 inhibitor for the preservation of peptide hormones (GLP-1 and PYY), and the other did not contain these additives. Blood was then centrifuged at 4000xg for 20 minutes following dissection in order to collect the blood serum from whole blood. The animal's gastrointestinal tract, stomach to anus, was then removed and stripped of mesenteric fat and then weighed filled with contents. This weight was used to subtract from the regular body weight (weight of the non-fasted rats on morning of killing) to determine the disemboweled body weight (DBW). The stomach, small intestine, cecum and the rest of the large intestine were each weighed with their contents. Then the contents were removed and the GI tract components reweighed empty. These empty component weights were added to the DBW to calculate an emboweled body weight (EBW). Ileal, cecal, and colonic contents were collected and stored in 1.5 ml microcentrifuge

tubes in liquid nitrogen before being transferred to a -80⁰C freezer. Epididymal, perirenal, and retroperitoneal fat pads used to calculate the abdominal fat content from the abdominal cavity were removed, placed in packets, and stored in liquid nitrogen until being transferred to a -80⁰C freezer. Full and empty cecal weights were recorded and analyzed for the effects of RS.

The pH of the ileal, cecal, and colonic contents was analyzed by a pH meter (Thermo Fisher Scientific) in thawed contents. Short chain fatty acid measurements of cecal samples were conducted by combining 0.5 grams of cecal contents, 1 ml of a 25% solution of metaphosphoric acid, and 4.5 ml of distilled water. Samples were then centrifuged and filtered and the effluent was analyzed by gas-liquid chromatography. The SCFA concentrations are expressed as the concentration (millimoles per gram of cecal contents), and the total production of SCFA's was calculated by multiplying the SCFA concentration by the weight of the cecal contents in order to obtain the millimoles of SCFA's in the entire cecal contents. The three major SCFA's; acetate, propionate, and butyrate; are expressed the same as the total amounts of SCFAs (2 carbon to 8 carbon SCFAs combined).

The total amount of glucagon-like peptide 1 (GLP-1), and GLP-1 active concentrations were measured in the serum using enzyme linked immunosorbent assay (ELISA) kits (ALPCO, Salem, NH). Serum peptide YY (PYY) was analyzed using a radioimmunoassay kit (Millipore, Billerica, MA)

Statistical Analysis

Data were analyzed using SAS 9.2. A 2X2X2 factorial arrangement of treatments (three-way ANOVA) was the statistical design of the study. Thus, there were 2 levels of three independent variables: RS (+ and -), fish oil (+ and -), and fat (high and low). Least Significant

Differences (LSD) mean comparison tests were also performed for all dependant variables. The results were considered significant at $p < 0.05$. Values are expressed as means \pm SE.

CHAPTER 4: RESULTS

Fermentation

As expected, two of the major markers of fermentation (pH and SCFA) revealed a significant decrease and increase, respectively, in the cecum of the RS fed groups. The ileal contents and contents from the rest of the large intestine of the RS fed groups was also decreased. This effect of feeding RS on pH of ileal, cecal and rest of large intestine contents was also observed in the high fat diet fed animals as there was no reduction of effect size with high-fat diets in pH, but the effect was moderated in the SCFA in the cecal contents. Our lab considers fermentation to have occurred when the pH value of the terminal portion of the small intestine, and large intestine is significantly reduced. The pH was significantly reduced (Figure 1) in the ileum ($p<0.0006$), cecal contents ($p<0.0001$), and colon ($p<0.0001$). The main location of fermentation appears to be the cecum as there is a greater increase in contents in the cecum compared to the rest of the large intestine (data not shown). The empty cecum weights revealed a significant increase in RS fed animals ($p<0.001$) and also a significant decrease from feeding a high-fat diet ($p<0.01$). This interaction was significant ($p<0.01$) demonstrating an attenuating effect from fat on the empty cecum weights (figure 2). This indicates increased growth of the major site of fermentation with RS diets.

The analysis of SCFA was conducted in cecal contents because this was the observed site of the greatest fermentation as determined by a greater increase in contents in the cecum compared to the rest of the large intestine (data not shown). The results of the SCFA in cecal contents revealed significant increases in total C2-C8 SCFA concentrations ($p<0.0001$) and in total production of C2-C8 SCFA's ($p<0.001$) in animals fed RS, however animals fed a high fat diet experienced a significant moderation ($p<0.001$) in total C2-C8 SCFA production.

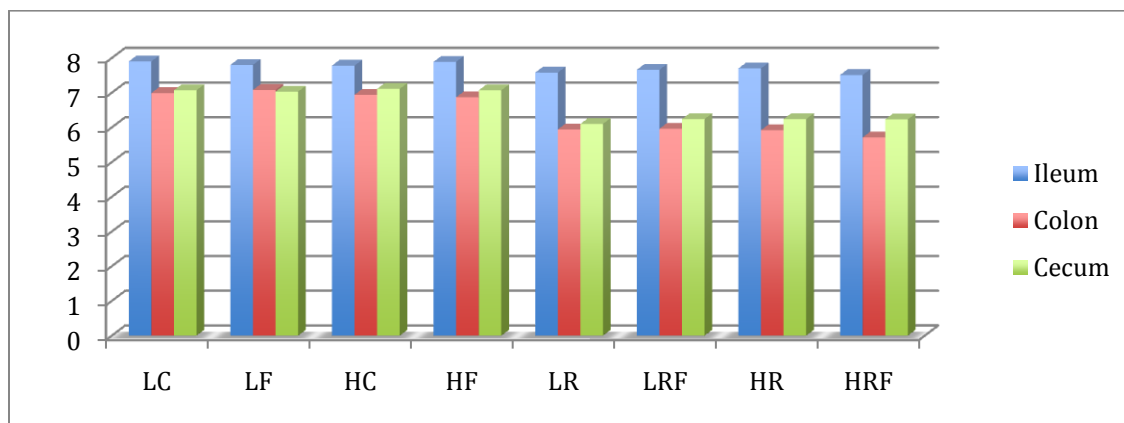


Figure 1: pH in Ileum, Colon, and Cecal contents.

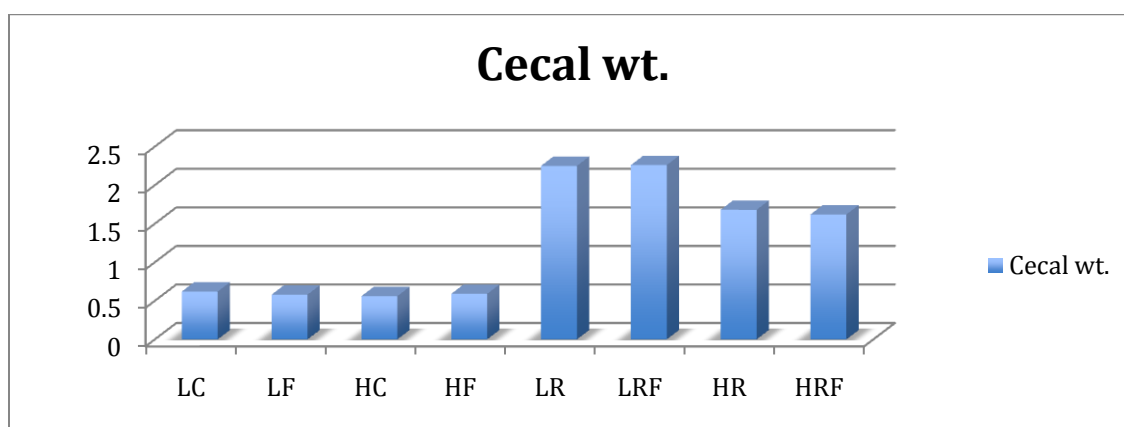


Figure 2: Empty Cecal weights (grams)

The interaction between RS and fat was also significant ($p < 0.007$) which demonstrates that the high-fat diet partially interferes with the RS effects on total production of C2-C8 SCFA's (Figures 3 and 4).

In the analysis of the specific SCFA's concentrations (millimoles/gram) in the cecal contents, a similar effect was observed in butyrate and acetate that was seen with SCFA total concentration, but this effect was not observed with propionate. The concentrations of acetate ($p < 0.0001$) and butyrate ($p < 0.0001$) were significantly increased in animals fed RS, but the high

fat diet resulted in a significant attenuation in the butyrate concentration ($p<0.001$). The interaction between fat and RS in specific SCFA concentrations was not significant. Propionate concentrations did not differ significantly for RS fed animals compared to animals not fed RS, but a trend towards an increase was observed ($p<0.15$). The results from the total amounts of specific SCFAs (millimoles) revealed that butyrate ($p<0.0001$), propionate ($p<0.001$), and acetate ($p<0.0001$) were each significantly increased in RS fed animals. Acetate ($p<0.01$) and butyrate ($p<0.001$) amounts were, however, significantly moderated in high fat fed animals, and the interactions between RS and fat were also significant for these SCFA's ($p<0.002$). These results reveal that RS is effective at increasing the total production and concentrations of butyrate and acetate, but the effects of a high fat diet reduce the amounts of these SCFA's in RS fed animals. A common effect of the high fat diet partially interfering (attenuating) with the effects of RS, but not eliminating the effects of RS, was observed in total amounts of butyrate, acetate, and C2-C8 SCFA. This effect was not observed for propionate. C2 to C8 totals and individual SCFA concentration (millimoles/gram) results can be seen in figure 3, and total production amounts (millimoles) in figure 4.

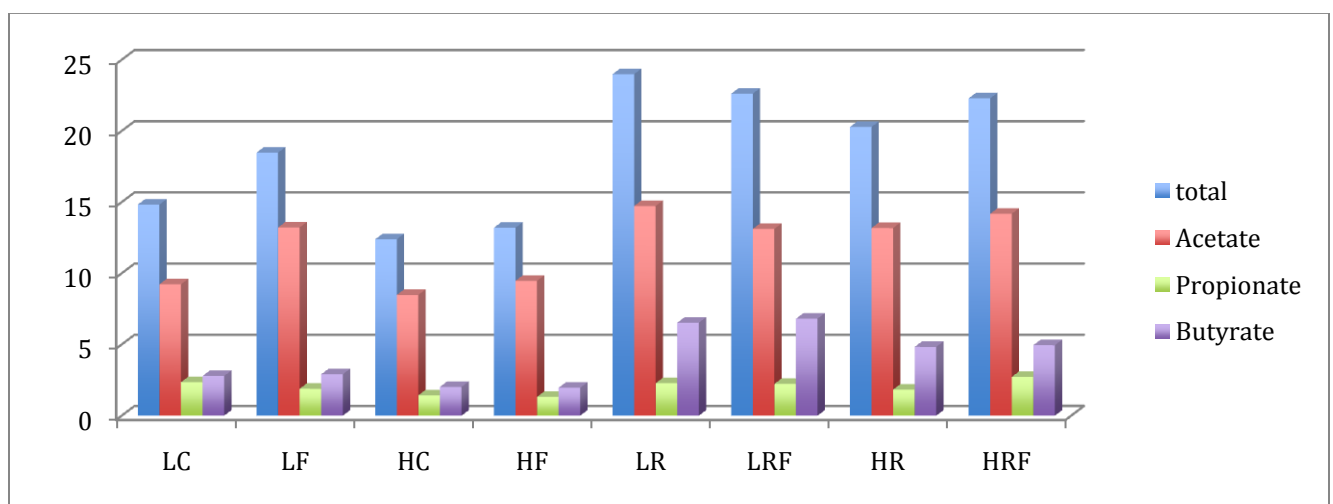


Figure 3: Cecal Contents of Combined C2 to C8 and Individual, acetate, propionate and butyrate, SCFA concentration (millimoles/g)

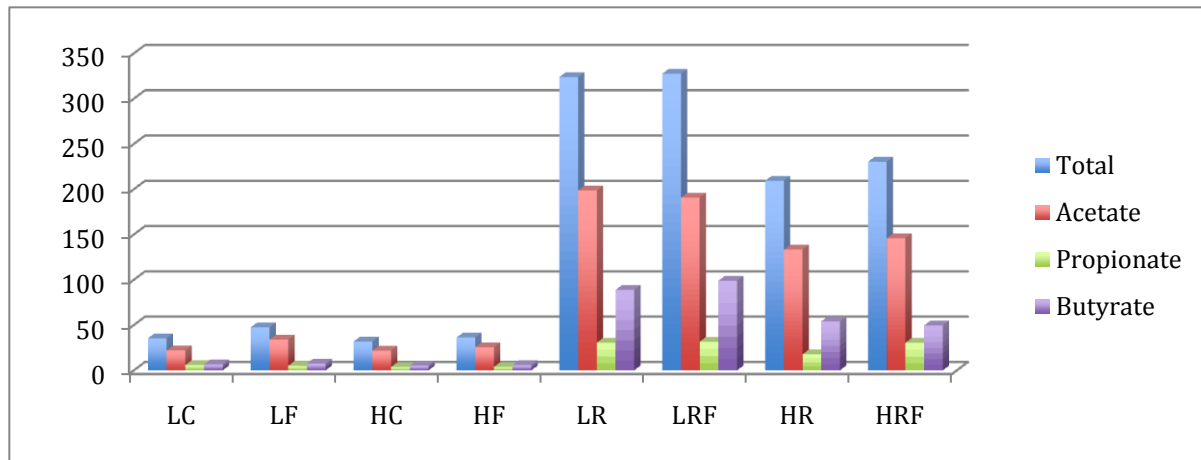


Figure 4: Cecal Contents of Total Production of Combined C2 to C8 SCFA and Individual, acetate, propionate and butyrate (millimoles)

Gut Hormones

The analysis of gut peptides revealed a significant increase in PYY ($p < 0.0001$), GLP-1 total ($p < 0.0001$), and GLP-1 active ($p < 0.0001$) in the RS fed groups (Figures 6, 7, and 8). The PYY results revealed the similar effect observed in millimoles for SCFA, butyrate, and acetate production. The high fat diet fed animals had an attenuated PYY concentration ($p < 0.001$), and a significant interaction between fat and RS ($p < 0.04$). This again demonstrates that a high fat diet partially interfered with the effects of RS, but did not eliminate the effects of RS stimulated PYY production

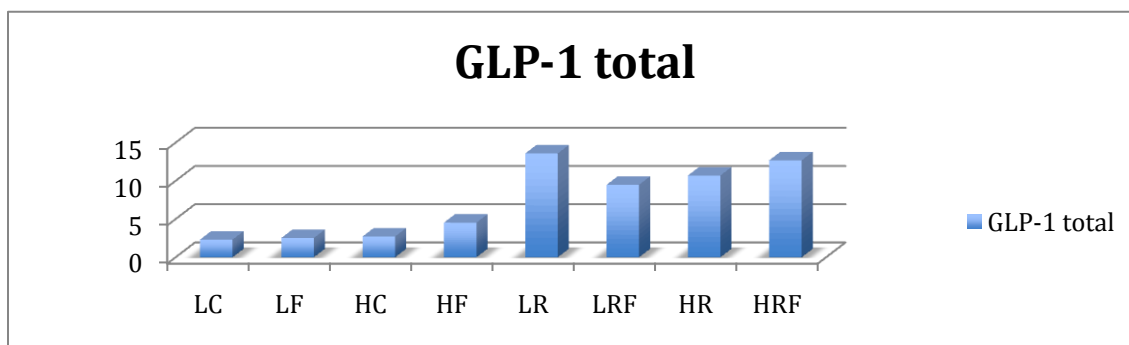


Figure 5: Glucagon like-peptide-1 total (GLP-1)

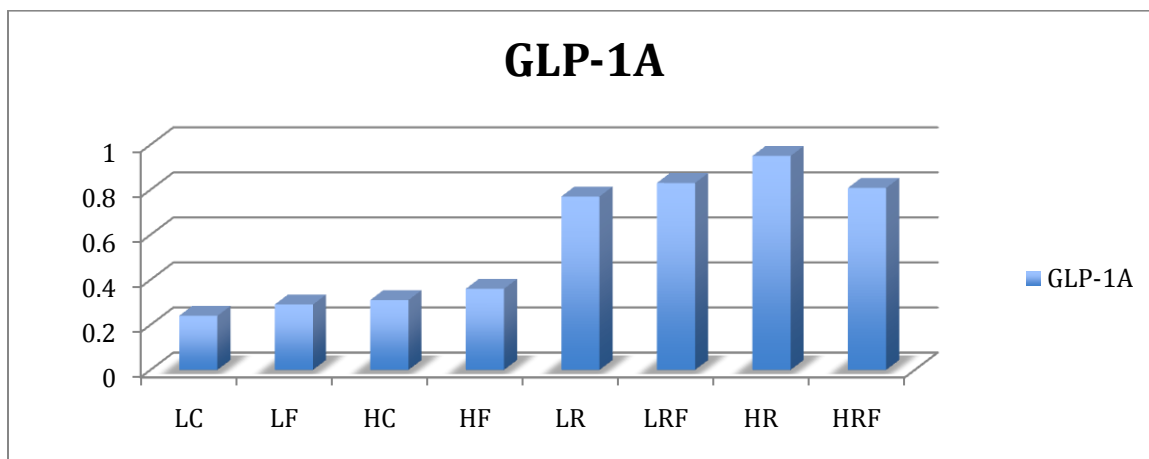


Figure 6: Glucagon like-peptide-1 active (GLP-1A)

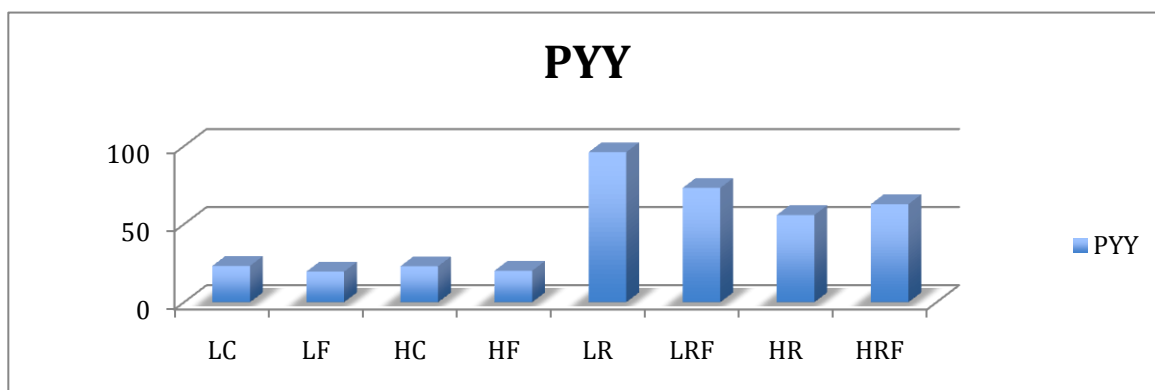


Figure 7: Peptide YY (PYY)

Body Composition

The disemboweled body weight (DBW) and emboweled body weights (EBW) were increased in high-fat fed groups ($p < 0.01$), but RS feeding only reduced the disemboweled body weights (DBW) ($p < 0.026$) (figure 8).

The results from the body weight and body fat analysis demonstrated a significant reduction in total abdominal fat/EBW percent ($p < 0.0008$) in RS groups, and a significant increase in high fat groups ($p < 0.0001$) (figure 9). However, there was not a significant interaction effect ($p = 0.18$), but a trend towards the common effect of the high fat diet partially interfering with the reduction in total abdominal fat/EBW percent observed overall with RS. Low-fat fed animals, which were fed RS, experienced a 24% reduction in abdominal fat/EBW when compared to the low-fat groups not fed RS, but high-fat fed groups only experienced a 9% reduction when compared to the high fat groups not fed RS (figure 9). Animals did not demonstrate a statistical difference in food intake within low and high fat diets, therefore, the effect on abdominal fat/EBW percent was not due to a difference in energy intakes in RS versus non-RS groups (figure 10). However, there was a significant fat effect ($p < 0.01$) in the total cumulative amount of food consumed with rats fed the high fat diet consuming less weight of food due to the higher energy density of the high-fat diet (figure 11).

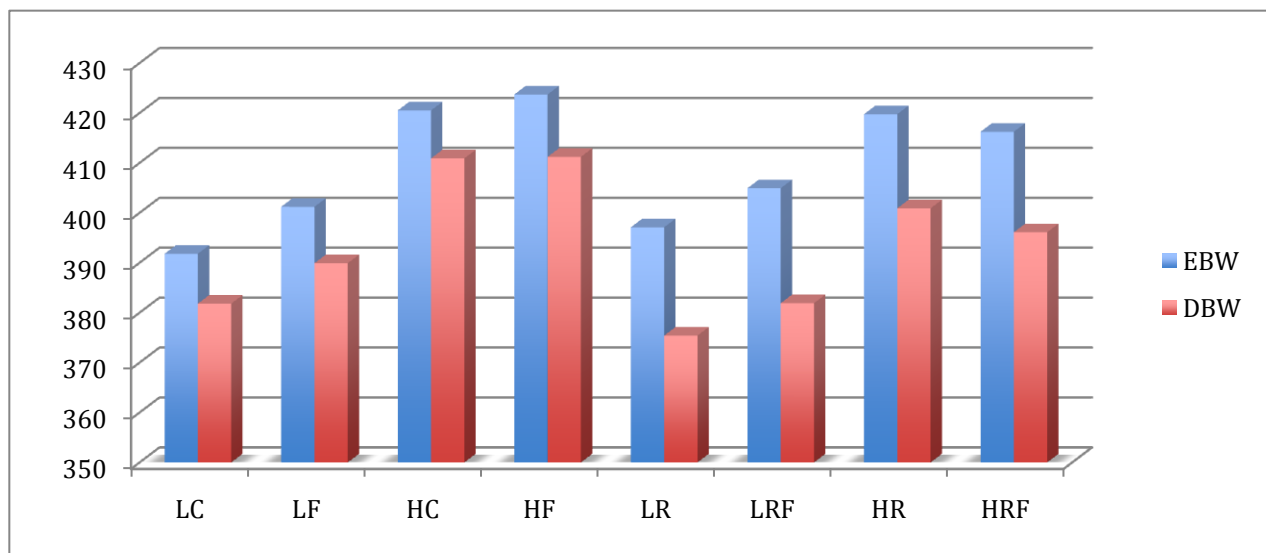


Figure 8: Emboweled Body Weight (EBW) and Disemboweled Body Weight (DBW)

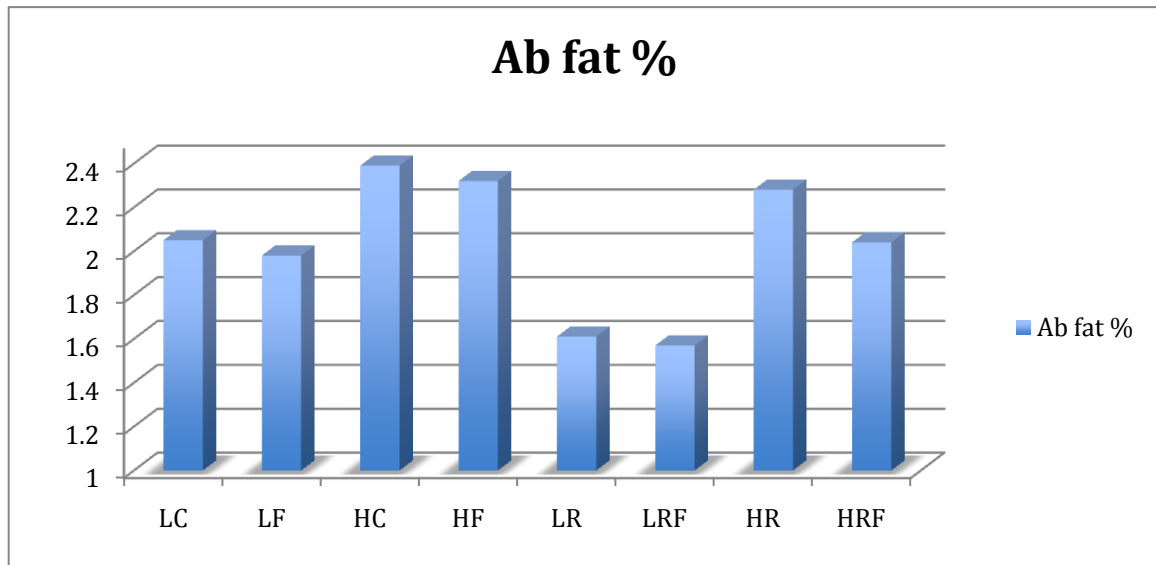


Figure 9: Abdominal fat %: Abdominal fat/Emboweled Body Weight

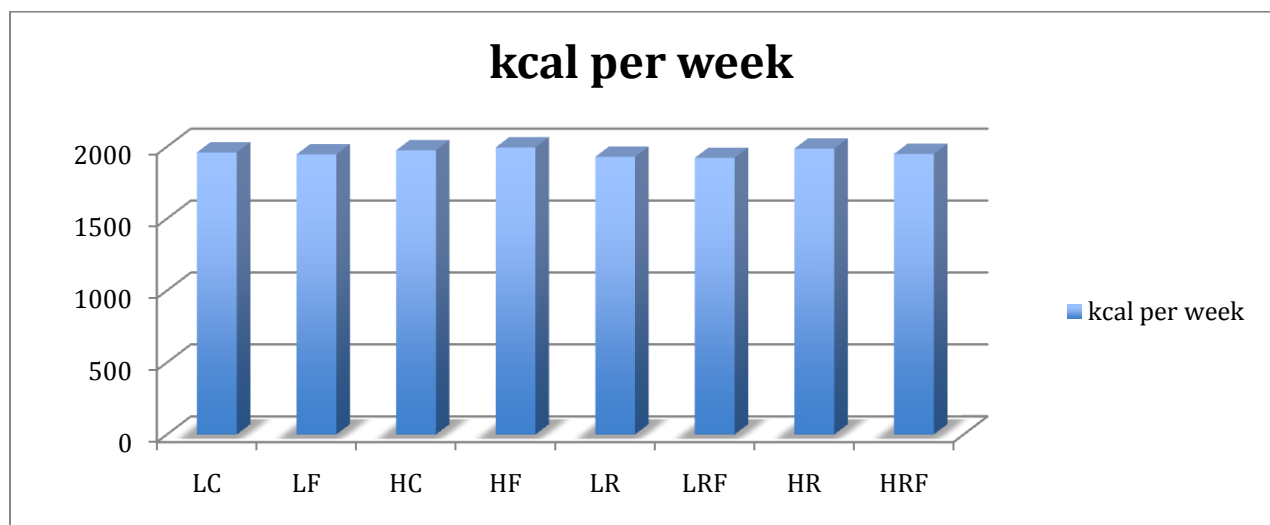


Figure 10: Food intake (average weekly intake in kilocalories)

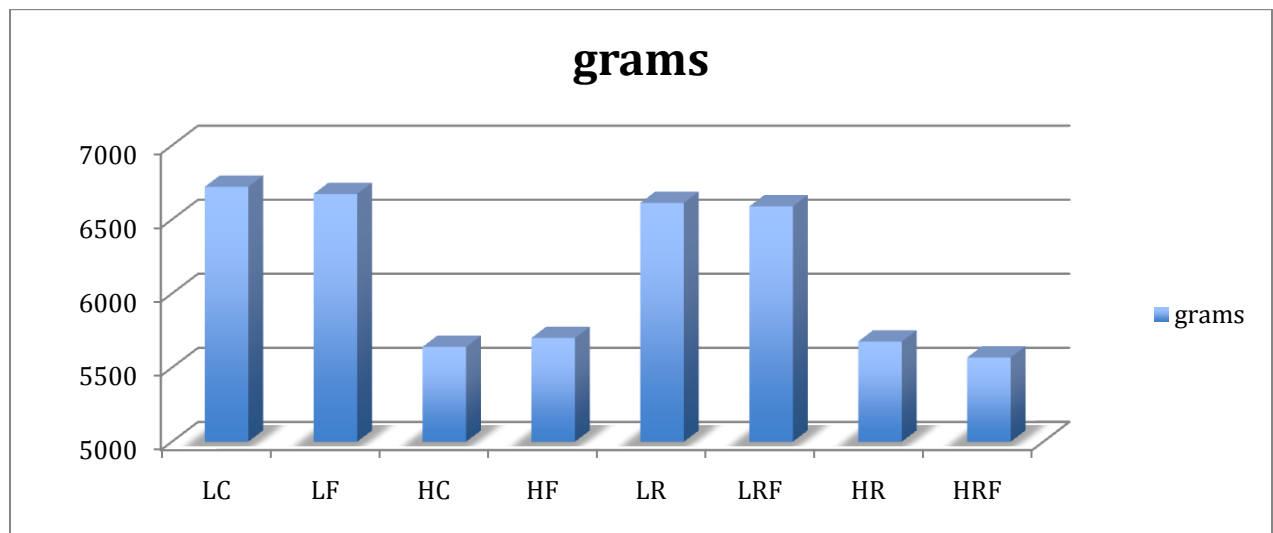


Figure 11: Cumulative food intake over 12 weeks (grams)

CHAPTER 5: DISCUSSION

The aim of this study was to assess the effects of adding a fermentable fiber source, RS, in high-fat and low-fat diets by measuring the effects on markers of fermentation, body weight and fat analyses, and two gut hormones of metabolic importance in rodents. Past research conducted by our lab has demonstrated positive prebiotic effects of fermentation and body fat loss through the addition of RS in the diet, but these effects are reduced or eliminated in animals fed high-fat diets prior to feeding RS. Feeding the high fat diet for several weeks to induce obesity before feeding RS was associated with no reduction in body fat in mice (Goldsmith et al 2010, Badkoobeh et al 2010). Feeding of a high fat diet at the same time as the feeding of RS in a short-term two week study also reduced bacteria that ferment the RS (unpublished data). It was hypothesized that the effect of RS when fed at the same time as high fat diet would have some beneficial effects on fermentation in the gut and some reduction of body fat. This was based on the fact that although fermentative bacteria were reduced in the cecum of mice fed a high fat diet with RS in a two week study, they were still present in the cecum at appreciably high levels. Thus, there may be a threshold effect with positive fermentation effects associated with feeding of a high fat diet at the same time as feeding RS. We had also hypothesized that the addition of fish oil into the diet containing the RS would restore the beneficial effects of RS observed in low-fat diets. This was hypothesized because our previous study with the combination of RS and fish oil in a low fat diet enhanced fermentation (Vidrine et al. 2009). Additionally, an *in vitro* study with a level of fat to mimic the amount that would reach the large intestine with the feeding of a high fat diet, demonstrated improved fermentation with fish oil compared to other types of fat, corn oil or lard (unpublished data).

In the current study, we observed beneficial effects of RS in both low and high fat diets, but a high fat diet partially interfered or attenuated some of the effects of RS that were observed in a low fat diet. While the results did not indicate a synergistic effect of fish oil and RS, further immune responses that will be measured in our lab, but not included in this thesis, may yet reveal some beneficial effects of fish oil.

Effects of Fermentation: pH and SCFA

The effects of fermentation, as determined by decreases in pH, indicate that the effect of RS addition in the diet can be observed throughout the terminal small intestine and large intestine of the GI tract. We focused on the ileum, cecum, and colonic portions of the GI tract and observed that, as expected, fermentation was increased in each of these areas, and was not reduced in the high fat fed animals.

A significant increase in total production (millimoles) of C2-C8 SCFA's, acetate, butyrate, and propionate was observed in RS fed animals. Increases in concentrations (millimoles/g) of C2-C8 SCFA's, butyrate, and acetate were observed in animals fed RS. High fat in the diet attenuated the total amounts (millimoles) of SCFA's and specifically butyrate and acetate. These results indicate that the addition of an RS source to the diet can create a beneficial GI health environment through the fermentation of the RS, and production of SCFA's in rats fed both low and high fat diets, but high fat in the diet can partially interfere with this effect.

Gut Hormones

The results from the gut hormone analysis suggest a relationship between increased markers of fermentation, decreased body fat percentage, and increased gut hormones. These results have also been observed in past research of our lab, and have allowed us to speculate to the causality of this relationship (Keenan et al 2006, Zhou et al 2008, Shen et al 2009). We

hypothesize that the increases in fermentation and SCFA production are playing a vital role in gut signaling pathways involved with the production of these hormones, which in turn exert anti-obesity effects in body fat loss. An *in vitro* study using primary cultures of cecal cells and cells from post cecal cells from the large intestine, demonstrated increased gene expression for proglucagon (gene for GLP-1) and PYY when cultured with acetate, butyrate, or propionate (Zhou et al. 2006).

The results from plasma peptide YY (PYY), total glucagon like peptide-1 (GLP-1), and GLP-1 active are in accordance with past studies in demonstrating a significant increase in these hormones in RS fed animals, however, the high fat diet did have a moderating effect in attenuating the PYY concentration.

Body Weight and Body Fat

The observed changes in body weight were quantified by using the total body weight (BW), disemboweled body weight (DBW), emboweled body weight (EBW), and abdominal fat percentage of emboweled body weight. The use of two common body composition analysis techniques, X-ray absorptiometry and NMR, used in our lab's previous studies to assess changes in body fat composition in rodents has largely been shown to be inaccurate (unpublished data). The results have not matched end of study excision of total abdominal fat (epididymal or uterine, perirenal and retroperitoneal). Our lab has found that quantification of body composition through the use of DBW, EBW, and abdominal fat as a percent of EBW has produced repeatable and more accurate results. Typically the size and weight of the GI tract and particularly the cecum with contents is greatly enlarged in animals fed RS, therefore the use of DBW and EBW allows us to more accurately control for this effect. The epididymal (uterine for female), perirenal, and

retroperitoneal fat pads are generally considered to be accurately correlated with total body fat percentage and have been used in past research (Keenan et al. 2006, Shen et al. 2009, Zhou et al. 2009, Shen et al. 2011).

The results from the study revealed that the animals fed the high fat diet had greater total, emboweled, and disemboweled body weights. The results from the DBW demonstrated that the addition of RS was associated with a significant decrease in body weight after the GI tract was removed. This result reveals that once the enlarged cecal weights with contents of the animals fed RS is accounted for through the removal of the GI tract; animals fed RS gained less weight. Animals demonstrated no significant difference in the calorie intake of food consumed within low or high fat diets, demonstrating that the effects on decreased body weight and body fat loss are not a result of decreased energy intake. However, high-fat fed animals did consume less total food amount due to a higher energy density of this diet. A previous study in mice using indirect calorimetry, demonstrated that feeding RS was associated with a decreased RQ (RER), which means a greater oxidation of fatty acids, and increased oxygen consumption, indicative of increased energy expenditure (Zhou et al. 2009).

The results of the reduced abdominal fat percentage (total abdominal fat/EBW) in RS fed rats compared to rats not fed RS demonstrated that RS was effective in low and high fat diets in reducing abdominal body fat. However, there was a trend ($p=0.18$) for attenuating the effect in high fat fed RS groups. This moderation of effect is suggested to be caused by the similar effect observed in PYY, C2-C8 SCFA's, butyrate, acetate, and the empty cecum weights. Thus, there are benefits of consuming RS with a high fat diet, but the benefits on body fat and GI tract health are attenuated compared to consuming RS with a lower fat diet. We know from our previous studies (unpublished data) and studies from other groups (Cani et al. 2008, de Lartigue et al.

2011, de La Serre et al. 2010) that the blunted effects of the RS in high fat groups may be because of alterations in the gut microbial community. The levels of fermentative bacteria are likely reduced in the current study, but still present in appreciable amounts to produce an effect, albeit reduced, with high fat diet feeding. Use of the factorial arrangement of treatments statistical design may also have given us greater statistical power to be able to detect smaller effect sizes with high fat diets that may not have been observed with a one-way ANOVA and *post hoc* t tests. These reduced beneficial health effects of RS with feeding of a high fat diet caused by reduction in bacterial populations, leads to the question of why no beneficial effect on reduction of abdominal body fat was observed in two of our previous studies in which mice were made obese with high fat diets for several weeks prior to the feeding of RS (Goldsmith et al. 2010, Badkoobeh et al. 2010). We speculate that high fat feeding without RS may change the bacterial populations so that the mice may have become non-responders to RS. More research is needed to confirm such speculation, but it is reported that there are non-responders to consumption of probiotics (beneficial bacteria) (Reid et al. 2010).

Another possible hypothesis for the reduction of effects of RS on abdominal body fat could be the result of the fat content interfering with signaling pathways involved with metabolism (de Lartigue et al. 2011). Signaling response to leptin is known to be reduced with feeding of a high fat diet (Lin et al. 2000), and GLP-1 signaling in the brain is reduced with feeding of a high fat diet (Nogueiras et al. 2009). .

Reflections

The results from the study suggest that the prebiotic effects of RS in the diet are enhanced in a low fat and high fat diet when compared to animals in the groups not fed RS. The fat content of the diet does affect the effectiveness of the RS at reducing body fat, but ultimately does

produce a significant reduction in body fat and other beneficial effects of RS. It is clear that a common theme within the study was that the high fat diet partially interfered or attenuated with the effectiveness of RS in SCFA production, particularly butyrate, PYY production, and a key marker of fermentation, cecal weight. There was a significant negative correlation between butyrate and abdominal fat percentage ($p < 0.003$) and PYY and abdominal fat percentage ($p < 0.007$) in the low fat fed animals. This significant correlation was lost in the high fat fed animals demonstrating that the effectiveness of dietary RS at lowering abdominal body fat is attenuated by the fat content in the diet and butyrate and PYY may be involved in the mechanism for this attenuation. This theme was also observed in the results of the abdominal body fat analysis. This gives further evidence to the hypothesis that fermentation of dietary fibers with the production of SCFA's and stimulation of gut hormones play an integral part in the observed effects on body fat reduction. The larger gut size and increased SCFA production in animals fed RS also suggest a two pronged beneficial effect of this fermentable fiber in promoting a healthier gut environment, which could be implicated in chronic disease etiology in the future.

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APPENDIX A : FISH OIL COMPOSITION

Saturated Fat (g) 14.4		144			Sodium Ascorbate (E301) 5.1895 51,895
Monounsaturated Fat (g) 11.7		117			Antioxidant Mixed Natural Tocopherols (E307b)* 0.1006
Polyunsaturated Fat (g) 18.5		185			Lecithin (E322) 0.0360 360
Trans Fat (g) 0.2		2			Antioxidant d l-alpha Tocopherol (E307c) 0.0024 24
Total Carbohydrates (g) 32.8	328	168	0.338144	0.173196	Antioxidant Ascorbyl Palmitate (E304) 0.0120 120
Sugars (g) 16		160	0	0.164948	Moisture Content (Water) 3.0000
Dietary Fibre (g) 0					Total 100.0000
Sodium (mg) 810	810				
Potassium (mg) 1.2	1.2				
Calcium (mg) 5.2	5.2				
micro	816.4				
Water Content (g) 3.0					
Ash (g) 1.4	14	14.43299			
	970	15.41856			

APPENDIX B: INSITUTIONAL ANIMAL CARE AND USE COMMITTEE



School of
Veterinary Medicine

Institutional Animal Care
And Use Committee

Division of
Laboratory Animal Medicine

Louisiana State University
Skip Bertman Drive
Baton Rouge, LA 70803

April 15, 2010

Dr. Michael Keenan
Human Ecology

Dear Dr. Keenan:

Protocol #10-023, entitled "Rescue of Fermentation of Resistant Starch in High Fat Diets with Fish Oil" lists you as the principal investigator.

I am happy to inform you that the above protocol is approved by the IACUC as of today, April 15, 2010. This approval is valid for 3 years, and authorizes the use of 108 rats.

Thank you.

Sincerely,

James E. Miller, DVM, MPVM, PhD
Chair

dbd

LSU

VITA

Jason Charrier has been a Louisiana resident his entire life. He received his bachelor's from the University of Louisiana at Lafayette with a concentration in Dietetics in the Fall of 2009. He attended Louisiana State University Graduate School during the Spring of 2010 and graduated with a masters degree in the department of human ecology in the summer of 2011. In the future, he plans to attend medical school and become an emergency room physician in Louisiana.